USE OF COLOSTRININ, CONSTITUENT PEPTIDES THEREOF, AND ANALOGS THEREOF AS MODULATORS OF INTRACELLULAR SIGNALING MOLECULES

This application claims priority to U.S. Provisional Application Serial No. 60/420,369, filed October 22, 2002, and is a Continuation-In-Part of U.S. Patent Application Serial No. 10/281,652, filed on October 28, 2002, which is a Divisional of U.S. Patent Application Serial No. 09/641,803, filed August 17, 2000 (issued on December 31, 2002 as U.S. Patent No. 6,500,798), which claims the benefit of U.S. Provisional Application Serial No. 60/149,310, filed August 17, 1999, all of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Colostrum is a component of the milk of mammals during the first few days after birth. Colostrum is a thick yellowish fluid and is the first lacteal secretion post parturition and contains a high concentration of immunogloblins (IgG, IgM, and IgA) and a variety of non-specific proteins. Colostrum also contains various cells such as granular and stromal cells, neutrophils, monocyte/macrophages, and lymphocytes. Colostrum also includes growth factors, hormones, and cytokines. Unlike mature breast milk, colostrum contains low sugar, low iron, but is rich is lipids, proteins, mineral salts, vitamins, and immunoglobins.

Colostrum also includes or contains a proline-rich polypeptide aggregate or complex, which is referred to as colostrinin (CLN). One peptide fragment of colostrinin is Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro (SEQ ID NO:31), which is disclosed in International Publication No. WO-A-98/14473. Colostrinin and this fragment have been identified as useful in the treatment of disorders of the

central nervous system, neurological disorders, mental disorders, dementia, neurodegenerative diseases, Alzheimer's disease, motor neurone disease, psychosis, neurosis, chronic disorders of the immune system, diseases with a bacterial and viral aetiology, and acquired immunological deficiencies, as set forth in International Publication No. WO-A-98/14473.

Although certain uses for colostrinin have been identified, it would represent an advancement in the art to discover and disclose other uses for colostrinin, or a component thereof, that are not readily ascertainable from the information currently known about colostrinin or its constituents.

SUMMARY OF THE INVENTION

The present invention relates to the use of colostrinin, at least one constituent (i.e., component) peptide thereof, at least one active analog thereof (e.g., peptide having an N-terminal sequence equivalent to an N-terminal sequence of at least one of the colostrinin constituent peptides), and combinations thereof, as modulators of intracellular signaling mechanisms. The signaling molecules discovered to date that are modulated include 4HNE adduct formation, GSH, P53, and JNK.

Furthermore, the present invention relates to the use of colostrinin, at least one constituent (i.e., component) peptide thereof, at least one active analog thereof (e.g., peptide having an N-terminal sequence equivalent to an N-terminal sequence of at least one of the colostrinin constituent peptides), and combinations thereof, in the inhibition of apoptosis. Specifically, the apoptotic (cytotoxic) effect of B amyloid on SH-SY5Y neuronal cells and TNF-alpha.

In one embodiment, the present invention provides a method of modulating an intracellular signaling molecule in a cell. The method includes contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide thereof, an active analog thereof, and combinations thereof, under conditions effective to accomplish at least one of the following: reduce 4HNE-protein adduct formation; inhibit 4HNE-mediated glutathione depletion; inhibit 4HNE-induced activation of p53 protein; or inhibit 4HNE-induced activation of c-Jun NH2-terminal kinases.

In one embodiment, the present invention provides a method of down regulating 4HNE-mediated lipid peroxidation in a cell. The method includes contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide thereof, an active analog thereof, and combinations thereof, wherein: the active analog is an active analog of a constituent peptide of colostrinin selected from the group of SEQ ID NO:1 through SEQ ID NO:34; the active analog comprises a peptide having an amino acid sequence with at least about 15 percent proline and having at least about 70 percent structural similarity to one or more constituent peptides of colostrinin; and the active analog does not interfere with cellular uptake of redox-sensitive 2',7'-dihydro-dichlorofluorescein-diacetate.

In one embodiment, the present invention provides a method for inhibiting apoptosis in a cell (typically, due to DNA damage). The method includes contacting the cell with an effective amount of an apoptosis inhibitor selected from the group of colostrinin, a constituent peptide thereof, an active analog thereof, and combinations thereof.

In another embodiment of inhibiting apoptosis in a cell, a method is provided that includes contacting the cell with an effective amount of an apoptosis inhibitor selected from the group of colostrinin, a constituent peptide thereof, an active analog thereof, and combinations thereof, wherein; the active analog is an active analog of a constituent peptide of colostrinin selected from the group of SEQ ID NO:1 through SEQ ID NO:34; the active analog comprises a peptide having an amino acid sequence with at least about 15 percent proline and having at least about 70 percent structural similarity to one or more constituent peptides of colostrinin; and the active analog does not interfere with cellular uptake of redox-sensitive 2',7'-dihydro-dichlorofluorescein-diacetate.

Other methods of the present invention include protecting against DNA damage in a cell, and reducing the toxic effect of β-amyloid or retinoic acid on a cell. These methods involve contacting the cell with an effective amount of a compound selected from the group of colostrinin, a constituent peptide thereof, an active analog thereof, and combinations thereof.

The cell can be present in a cell culture, a tissue, an organ, or an organism. For certain embodiments, the cell is a mammalian cell. For certain embodiments, the cell is a human cell.

For certain embodiments, the compound (e.g. modulator such as an apoptosis inhibitor) is a constituent peptide of colostrinin. Preferably, the modulator is selected from the group of MQPPPLP (SEQ ID NO:1), LQTPQPLLQVMMEPQGD (SEQ ID NO:2), DQPPDVEKPDLQPFQVQS (SEQ ID NO:3), LFFFLPVVNVLP (SEQ ID NO:4), DLEMPVLPVEPFPFV (SEQ ID NO:5), MPQNFYKLPQM (SEQ ID NO:6), VLEMKFPPPPQETVT (SEQ ID NO:7), LKPFPKLKVEVFPFP (SEQ ID NO:8), VVMEV (SEQ ID NO:9), SEQP (SEQ ID NO:10), DKE (SEQ ID NO:11), FPPPK (SEQ ID NO:12), DSQPPV (SEQ ID NO:13), DPPPPQS (SEQ ID NO:14), SEEMP (SEQ ID NO:15), KYKLQPE (SEQ ID NO:16), VLPPNVG (SEQ ID NO:17), VYPFTGPIPN (SEQ ID NO:18), SLPQNILPL (SEQ ID NO:19), TQTPVVVPPF (SEQ ID NO:20), LQPEIMGVPKVKETMVPK (SEQ ID NO:21), HKEMPFPKYPVEPFTESQ (SEQ ID NO:22), SLTLTDVEKLHLPLPLVQ (SEQ ID NO:23), SWMHQPP (SEQ ID NO:24), QPLPPTVMFP (SEQ ID NO:25), PQSVLS (SEQ ID NO:26), LSQPKVLPVPQKAVPQRDMPIQ (SEQ ID NO:27), AFLLYQE (SEQ ID NO:28), RGPFPILV (SEQ ID NO:29), ATFNRYQDDHGEEILKSL (SEQ ID NO:30), VESYVPLFP (SEQ ID NO:31), FLLYQEPVLGPVR (SEQ ID NO:32), LNF (SEQ ID NO:33), and MHQPPQPLPPTVMFP (SEQ ID NO:34), and combinations thereof.

As used herein, "a" or "an" means one or more (or at least one), such that combinations of active agents (i.e., active oxidative stress regulators), for example, can be used in the compositions and methods of the invention. Thus, a composition that includes "a" polypeptide refers to a composition that includes one or more polypeptides.

"Amino acid" is used herein to refer to a chemical compound with the general formula: NH_2---CRH---COOH, where R, the side chain, is H or an organic group. Where R is organic, R can vary and is either polar or nonpolar (i.e., hydrophobic). The amino acids of this invention can be naturally occurring or synthetic (often referred to as nonproteinogenic). As used herein,

an organic group is a hydrocarbon group that is classified as an aliphatic group, a cyclic group or combination of aliphatic and cyclic groups. The term "aliphatic group" means a saturated or unsaturated linear or branched hydrocarbon group. This term is used to encompass alkyl, alkenyl, and alkynyl groups, for example. The term "cyclic group" means a closed ring hydrocarbon group that is classified as an alicyclic group, aromatic group, or heterocyclic group. The term "alicyclic group" means a cyclic hydrocarbon group having properties resembling those of aliphatic groups. The term "aromatic group" refers to mono- or polycyclic aromatic hydrocarbon groups. As used herein, an organic group can be substituted or unsubstituted.

The terms "polypeptide" and "peptide" are used interchangeably herein to refer to a polymer of amino acids. These terms do not connote a specific length of a polymer of amino acids. Thus, for example, the terms oligopeptide, protein, and enzyme are included within the definition of polypeptide or peptide, whether produced using recombinant techniques, chemical or enzymatic synthesis, or naturally occurring. This term also includes polypeptides that have been modified or derivatized, such as by glycosylation, acetylation, phosphorylation, and the like.

The following abbreviations are used throughout the application:

A = Ala = Alanine	T = Thr = Threonine
V = Val = Valine	C = Cys = Cysteine
L = Leu = Leucine	Y = Tyr = Tyrosine
I = Ile = Isoleucine	N = Asn = Asparagine
P = Pro = Proline	Q = Gln = Glutamine
F = Phe = Phenylalanine	D = Asp = Aspartic Acid
W = Trp = Tryptophan	E = Glu = Glutamic Acid
M = Met = Methionine	K = Lys = Lysine
G = Gly = Glycine	R = Arg = Arginine

S = Ser = Serine	H = His = Histidine

BRIEF DESCRIPTION OF THE DRAWINGS

The invention can be better understood with reference to the following detailed description together with the appended illustrative drawings in which like elements are numbered the same:

Figure 1. Colostrinin inhibits formation of protein-HNE (i.e., 4-HNE protein) adducts. (A): 4HNE (25 nM); (B): H_2O_2 (100 μM); (C): CLN(10 μg/ml) pre-treatment followed by 4HNE (25 nM) exposure; (D): LAH (10 μg/ml) pre-treatment followed by 4HNE (25 nM) exposure; (E): HNE-protein adducts detected by Western blot analysis. Lane 1, 25 nM; lane 2, 12.5 nM; lane 3, 6.2 nM of 4HNE alone; lanes 4-6, CLN (10 μg/ml) plus 4HNE, 25, 12.5 and 6.2 nM, respectively.

Figure 2. Colostrinin inhibits 4HNE-induced oxidative stress. (A): 1, control; 2, colostrinin (10 μg/ml); 3, 4HNE (25 nM); 4, 4HNE (25 nM) plus colostrinin (10 μg/ml); 5, lactalbumin hydrolysate (10 μg/ml); 6, lactalbumin hydrolysate (10 μg/ml) plus 4HNE (25 nM). (B): A representative FACS histogram of fluorescence of cells treated with 4HNE (25 nM) and CLN (10 μg/ml) plus 4HNE.

Figure 3. Effect of CLN on 4HNE-induced loss of intracellular GSH levels. Cells were mock-treated or treated with CLN (or LAH) and/or 4HNE for 30 min, and o-phthalaldehyde-mediated fluorescence was determined as described in Materials and Methods. Open columns: 1, mock-treated; 2, CLN (10 μg/ml)-; 3, LAH (10 μg/ml)-; 4, 4HNE (25 nM)-treated. Filled solid columns: 5, CLN (10 μg/ml) pre- and 4HNE (25 nM)-treated for 30 min; 6, LAH (10 μg/ml) pre- and 4HNE (25 nM)-treated for 30 min.

Figure 4. Inhibition of JNK induction by colostrinin. A change in JNK's phosphotyrosine levels was monitored by SDS-PAGE analysis. Equal amounts of protein (50 μg) were fractionated, blotted, and probed with anti-phospho- (Thr-183/Tyr-185)- JNK antibody. Lanes 1 and 2, mock-treated cells; lane 3, 8-(4-chlorophenylthio)-cAMP, an inhibitor of JNK activation; lanes 4 and 5, 25 nM 4HNE; lane 6, CLN (10 μg/ml) alone; lane 7, 25 nM 4HNE plus

10 μg/ml CLN; lane 8, 25 nM 4HNE plus 1 μg/ml CLN; lane 9, 25 nM 4HNE plus 0.1 μg/ml CLN.

Figure 5. CLN reduces 4HNE-mediated activation of p53. PC12 cells were pre-treated with CLN or LAH and exposed to 4HNE. Three hours after treatment, cell lysates were analyzed by Western blot analysis. (B) p53; (A) corresponding α-tubulin. 4HNE (25 nM), CLN (10 μg/ml), LAH (10 μg/ml).

Figures 6A-6D. (A) Normal morphology of SH-SY5Y control cells. Cells are mostly clumped, non-contact inhibited (right arrow) with a few elongated cells present. Their refractability indicates they are healthy and growing normally. (B) Cells treated with Beta-amyloid (10 μg/ml added on day 5) that show its toxicity. Note small round granulated cells with little refractability. (C) Differentiated SH-SY5Y cells following treatment with CLN (0.1 μg/ml added on day 5 for 30 minutes). Touching cells are flat, contact inhibited (not clumped), left arrow, and more isolated cells are elongated and neuronal in appearance, right arrow. (D) Cells protected from toxic (apoptotic effect) of Beta-amyloid by treating with CLN (Colostrinin 0.1 μg/ml added on day 5 for 30 minutes + Beta-amyloid 10 μg/ml added on day 5). Cells are flat (upper arrow) or elongated (lower arrow) showing typical morphology of differentiated cells (see Fig. 6C). (E) Inhibition of toxicity (apototic activity) of Beta-amyloid by CLN treatment (Colostrinin 3 μg/ml added on day 5 for 30 minutes + Beta-amyloid 10 μg/ml added on day 5). Note flattened (bottom arrow) and elongated (upper arrow) cells typical of SH-SY5Y differentiated cells. (F) Toxic (apoptotic) effect of retinoic acid (20 μM added on day 1) on SH-SY5Y cells. The observed toxicity resembles cytopathology induced by viruses. Cytoplasmic bridging caused by shrinking of cells once in contact with each other (upper right arrows), shrunken granular cells (lower right arrow) and small round cells (lower left arrows). (G) Inhibition of toxic effect of retinoic acid by treatment of SH-SY5Y with CLN (20 μM retinoic acid added on day 1 + 1 μg/ml Colostrinin added on day 5 for 30 minutes). Cells are well organized showing typical morphology of differentiated SH-SY5Y cells, elongation (lower arrow) and flattening (upper arrow).

Figure 7. Analysis of apoptosis by flow cytometry. (A) Induction of apoptosis by 4HNE (100 nM). UL, upper left; UR, upper right: necrotic cells;

LL, lower left: viable cells; LR, lower right: apoptotic cells. (B) Absence of apoptosis in mock-treated cells. UL, upper left; UR, upper right: necrotic cells; LL, lower left: viable cells; LR, lower right: apoptotic cells.

Figure 8. Inhibition of 4HNE-induced apoptosis by CLN. PC12 cells were treated with CLN (1 μg per ml) for 15 min and 4HNE (100 nM) was added. Twenty four hours later, cells were harvested and stained with annexin V-PE and 7-AAD. 1, solvent alone; 2, 100 nM 4HNE; 3, TROLOX (vitamin E) 4, col (internal control) + 100 nM 4HNE; 5, CLN alone (1 μg per ml); 6, CLN (1 μg per ml) + 100 nM 4HNE.

Figure 9. Inhibition of UV-B-induced apoptosis by CLN. Parallel cultures of PC12 cells were treated with CLN (1 μg per ml) or col (1 μg per ml) and exposed to LD50 of UV-B. Twenty four hours later, cells were harvested and stained with annexin V-PE and 7-AAD. 1, Mock-treated; 2, UV-B (LD50); 3, col (internal control); 4, col + UV-B (LD50); 5, CLN alone (1 μg per ml); 6, CLN (1 μg per ml) + UV-B (LD50).

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Colostrinin, a complex of proline-rich polypeptides derived from ovine colostrum, induces mitogenic stimulation and a variety of cytokines in human peripheral blood leukocytes. It also possesses anti-oxidant activity in pheochromocytoma (PC12) cells.

It has been discovered that colostrinin, at least one constituent peptide thereof, and/or at least one active analog thereof (e.g., a peptide having an N-terminal sequence equivalent to an N-terminal sequence of at least one of the colostrinin constituent peptides) can be used as modulators of intracellular signaling mechanisms. The signaling molecules discovered to date that are modulated include 4HNE adduct formation, GSH, P53, and JNK.

More specifically, the present invention provides methods that involve: 1) reduction of the abundance of 4HNE-protein adducts as shown by fluorescent microscopy and Western blot analysis; 2) reduction of intracellular levels of ROS as shown by a decrease in 2',7'dichlorodihydro-fluorescein-mediated fluorescence; 3) inhibition of 4HNE-mediated glutathione depletion as determined fluorimetrically; and 4) inhibition of 4HNE-induced activation of c-

Jun NH2-terminal kinases. Furthermore, the present invention provides methods that down regulate the 4HNE-mediated lipid peroxidation and its product-induced signaling that otherwise may lead to pathological changes at the cellular and organ level.

Also, the present invention relates to the use of colostrinin, at least one constituent (i.e., component) peptide thereof, at least one active analog thereof (e.g., peptide having an N-terminal sequence equivalent to an N-terminal sequence of at least one of the colostrinin constituent peptides), and combinations thereof, in the inhibition of apoptosis, specifically, the inhibition is related to the apoptotic (cytotoxic) effect of β-amyloid on SH-SY5Y neuronal cells and TNF-alpha or the apoptotic effect of retinoic acid.

Such compounds (e.g. modulators such as inhibitors) are referred to herein as "active agents." Significantly, such active agents can be administered alone or in various combinations to a patient (e.g., animals including humans) as a medication or dietary (e.g., nutrient) supplement in a dose sufficient to produce the desired effect throughout the patient's body, in a specific tissue site, or in a collection of tissues (organs).

Colostrinin is composed of peptides, the aggregate of which has a molecular weight range between about 5.8 to about 26 kiloDaltons (kDa) determined by polyacrylamide gel electrophoresis. It has a greater concentration of proline than any other amino acid. Ovine colostrinin has been found to have a molecular weight of about 18 kDa and includes three non-covalently linked subunits having a molecular weight of about 6 kDa and has about 22 wt-% proline.

Colostrinin has been found to include a number of peptides ranging from 3 amino acids to 22 amino acids or more. These can be obtained by various known techniques, including isolation and purification involving eletrophoresis and synthetic techniques. The specific method of obtaining colostrinin and SEQ ID NO:31 is described in International Publication No. WO 98/14473. Using HPLC and Edelman Degradation, over 30 constituent peptides of colostrinin have been identified, which can be classified into several groups: (A) those of unknown precursor; (B) those having a β-casein homologue precursor; (C) those having a β-casein precursor; and (D) those having an

annexin precursor. These peptides are described in International Patent Publication No. WO 00/75173, published December 14, 2000, and can be synthesized according to well-known synthetic methods. These peptides (i.e., constituent peptides of colostrinin), which can be derived from colostrinin or chemically synthesized, include: MQPPPLP (SEQ ID NO:1); LQTPQPLLQVMMEPQGD (SEQ ID NO:2); DQPPDVEKPDLQPFQVQS (SEQ ID NO:3); LFFFLPVVNVLP (SEQ ID NO:4); DLEMPVLPVEPFPFV (SEQ ID NO:5); MPQNFYKLPQM (SEQ ID NO:6); VLEMKFPPPPQETVT (SEQ ID NO:7); LKPFPKLKVEVFPFP (SEQ ID NO:8); VVMEV (SEQ ID NO:9); SEQP (SEQ ID NO:10); DKE (SEQ ID NO:11); FPPPK (SEQ ID NO:12); DSQPPV (SEQ ID NO:13); DPPPPQS (SEQ ID NO:14); SEEMP (SEQ ID NO:15); KYKLQPE (SEQ ID NO:16); VLPPNVG (SEQ ID NO:17); VYPFTGPIPN (SEQ ID NO:18); SLPQNILPL (SEQ ID NO:19); TQTPVVVPPF (SEQ ID NO:20); LQPEIMGVPKVKETMVPK (SEQ ID NO:21); HKEMPFPKYPVEPFTESQ (SEQ ID NO:22); SLTLTDVEKLHLPLPLVQ (SEQ ID NO:23); SWMHQPP (SEQ ID NO:24); QPLPPTVMFP (SEQ ID NO:25); PQSVLS (SEQ ID NO:26); LSQPKVLPVPQKAVPQRDMPIQ (SEQ ID NO:27); AFLLYQE (SEQ ID NO:28); RGPFPILV (SEQ ID NO:29); ATFNRYQDDHGEEILKSL (SEQ ID NO:30); VESYVPLFP (SEQ ID NO:31); FLLYQEPVLGPVR (SEQ ID NO:32); LNF (SEQ ID NO:33); and MHQPPQPLPPTVMFP (SEQ ID NO:34). These can be classified as follows: (A) those of unknown precursor include SEQ ID NOs:2, 6, 7, 8, 10, 11, 14, and 33; (B) those having a β-casein homologue precursor include SEQ ID NOs:1, 3, 4, 5, 9, 12, 13, 15, 16, 17, and 31; (C) those having a β-casein precursor include SEQ ID NOs:18 (casein amino acids 74-83), 19 (casein amino acids 84-92), 20 (casein amino acids 93-102), 21 (casein amino acids 103-120), 22 (casein amino acids 121-138), 23 (casein amino acids 139-156), 24 (casein amino acids 157-163), 25 (casein amino acids 164-173), 26 (casein amino acids 174-179), 27 (casein amino acids 180-201), 28 (casein amino acids 202-208), 29 (casein amino acids 214-222), 32 (casein amino acids 203-214), and 34 (casein amino acids 159-173); and (D) those having an annexin precursor include SEQ ID NO:30 (annexin amino acids 203-220).

A preferred group of such peptides includes: MQPPPLP (SEQ ID NO:1); LQTPQPLLQVMMEPQGD (SEQ ID NO:2); DQPPDVEKPDLQPFQVQS (SEQ ID NO:3); LFFFLPVVNVLP (SEQ ID NO:4); DLEMPVLPVEPFPFV (SEQ ID NO:5); MPQNFYKLPQM (SEQ ID NO:6); VLEMKFPPPPQETVT (SEQ ID NO:7); LKPFPKLKVEVFPFP (SEQ ID NO:8); and combinations thereof.

The polypeptides of SEQ ID NOs:1-34 can be in their free acid form or they can be amidated at the C-terminal carboxylate group. The present invention also includes analogs of the polypeptides of SEQ ID NOs:1-34, which includes polypeptides having structural similarity with SEQ ID NOs:1-34. These peptides can also form a part of a larger peptide. An "analog" of a polypeptide includes at least a portion of the polypeptide, wherein the portion contains deletions or additions of one or more contiguous or noncontiguous amino acids, or containing one or more amino acid substitutions. An "analog" can thus include additional amino acids at one or both of the terminii of the polypeptides listed above. Substitutes for an amino acid in the polypeptides of the invention are preferably conservative substitutions, which are selected from other members of the class to which the amino acid belongs. For example, it is well-known in the art of protein biochemistry that an amino acid belonging to a grouping of amino acids having a particular size or characteristic (such as charge, hydrophobicity and hydrophilicity) can generally be substituted for another amino acid without substantially altering the structure of a polypeptide.

For the purposes of this invention, conservative amino acid substitutions are defined to result from exchange of amino acids residues from within one of the following classes of residues: Class I: Ala, Gly, Ser, Thr, and Pro (representing small aliphatic side chains and hydroxyl group side chains); Class II: Cys, Ser, Thr and Tyr (representing side chains including an -OH or -SH group); Class III: Glu, Asp, Asn and Gln (carboxyl group containing side chains): Class IV: His, Arg and Lys (representing basic side chains); Class V: Ile, Val, Leu, Phe and Met (representing hydrophobic side chains); and Class VI: Phe, Trp, Tyr and His (representing aromatic side chains). The classes also include related amino acids such as 3Hyp and 4Hyp in Class I; homocysteine in Class II; 2-aminoadipic acid, 2-aminopimelic acid, (γ-carboxyglutamic acid, β-

carboxyaspartic acid, and the corresponding amino acid amides in Class III; ornithine, homoarginine, N-methyl lysine, dimethyl lysine, trimethyl lysine, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, homoarginine, sarcosine and hydroxylysine in Class IV; substituted phenylalanines, norleucine, norvaline, 2-aminooctanoic acid, 2-aminoheptanoic acid, statine and β-valine in Class V; and naphthylalanines, substituted phenylalanines, tetrahydroisoquinoline-3-carboxylic acid, and halogenated tyrosines in Class VI.

Preferably, the active analogs of colostrinin and its constituent peptides include polypeptides having a relatively large number of proline residues. Because proline is not a common amino acid, a "large number" preferably means that a polypeptide includes at least about 15% proline (by number), and more preferably at least about 20% proline (by number). Most preferably, active analogs include more proline residues than any other amino acid.

As stated above, active analogs of colostrinin and its constituent peptides include polypeptides having structural similarity. Structural similarity is generally determined by aligning the residues of the two amino acid sequences to optimize the number of identical amino acids along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of identical amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. Preferably, two amino acid sequences are compared using the Blastp program, version 2.0.9, of the BLAST 2 search algorithm, available at http://www.ncbi.nlm.nih.gov/gorf/bl2.html. Preferably, the default values for all BLAST 2 search parameters are used, including matrix = BLOSUM62; open gap penalty = 11, extension gap penalty = 1, gap x_dropoff = 50, expect = 10, wordsize = 3, and filter on. In the comparison of two amino acid sequences using the BLAST search algorithm, structural similarity is referred to as "identity." Preferably, an active analog of colostrinin or its constituent peptides has a structural similarity to colostrinin or one or more of its constituent peptides (preferably, one of SEQ ID NOs:1-34) of at least about 70% identity, more preferably, at least about 80% identity, and most preferably, at least about 90% identity.

Colostrinin or any combination of its peptide components or active analogs thereof can be derived (preferably, isolated and purified) naturally such as by extraction from colostrum or can be synthetically constructed using known peptide polymerization techniques. For example, the peptides of the invention may be synthesized by the solid phase method using standard methods based on either t-butyloxycarbonyl (BOC) or 9-fluorenylmethoxy-carbonyl (FMOC) protecting groups. This methodology is described by G.B. Fields et al. in Synthetic Peptides: A User's Guide, W.M. Freeman & Company, New York, NY, pp. 77-183 (1992). Moreover, gene sequence encoding the colostrinin peptides or analogs thereof can be constructed by known techniques such as expression vectors or plasmids and transfected into suitable microorganisms that will express the DNA sequences thus preparing the peptide for later extraction from the medium in which the microorganism are grown. For example, U.S. Patent No. 5,595,887 describes methods of forming a variety of relatively small peptides through expression of a recombinant gene construct coding for a fusion protein which includes a binding protein and one or more copies of the desired target peptide. After expression, the fusion protein is isolated and cleaved using chemical and/or enzymatic methods to produce the desired target peptide.

The peptides used in the methods of the present invention may be employed in a monovalent state (i.e., free peptide or a single peptide fragment coupled to a carrier molecule). The peptides may also be employed as conjugates having more than one (same or different) peptide fragment bound to a single carrier molecule. The carrier may be a biological carrier molecule (e.g., a glycosaminoglycan, a proteoglycan, albumin or the like) or a synthetic polymer (e.g., a polyalkyleneglycol or a synthetic chromatography support). Typically, ovalbumin, human serum albumin, other proteins, polyethylene glycol, or the like are employed as the carrier. Such modifications may increase the apparent affinity and/or change the stability of a peptide. The number of peptide fragments associated with or bound to each carrier can vary, but from about 4 to 8 peptides per carrier molecule are typically obtained under standard coupling conditions.

For instance, peptide/carrier molecule conjugates may be prepared by treating a mixture of peptides and carrier molecules with a coupling agent, such as a carbodiimide. The coupling agent may activate a carboxyl group on either the peptide or the carrier molecule so that the carboxyl group can react with a nucleophile (e.g., an amino or hydroxyl group) on the other member of the peptide/carrier molecule, resulting in the covalent linkage of the peptide and the carrier molecule. For example, conjugates of a peptide coupled to ovalbumin may be prepared by dissolving equal amounts of lyophilized peptide and ovalbumin in a small volume of water. In a second tube, 1-ethyl-3-(3-dimethylamino-propyl)-carboiimide hydrochloride (EDC; ten times the amount of peptide) is dissolved in a small amount of water. The EDC solution was added to the peptide/ovalbumin mixture and allowed to react for a number of hours. The mixture may then dialyzed (e.g., into phosphate buffered saline) to obtain a purified solution of peptide/ovalbumin conjugate. Peptide/carrier molecule conjugates prepared by this method typically contain about 4 to 5 peptides per ovalbumin molecule.

The present invention also provides a composition that includes one or more active agents (i.e., colostrinin, at least one constituent peptide thereof, or active analog thereof) of the invention and one or more carriers, preferably a pharmaceutically acceptable carrier. The methods of the invention include administering to, or applying to the skin of, a patient, preferably a mammal, and more preferably a human, a composition of the invention in an amount effective to produce the desired effect. The active agents of the present invention are formulated for enteral administration (oral, rectal, *etc.*) or parenteral administration (injection, internal pump, *etc.*). The administration can be via direct injection into tissue, interarterial injection, intervenous injection, or other internal administration procedures, such as through the use of an implanted pump, or via contacting the composition with a mucus membrane in a carrier designed to facilitate transmission of the composition across the mucus membrane such as a suppository, eye drops, inhaler, or other similar administration method or via oral administration in the form of a syrup, a liquid, a pill, capsule, gel coated tablet, or other similar oral administration method. The active agents can be incorporated into an adhesive plaster, a patch, a gum,

and the like, or it can be encapsulated or incorporated into a bio-erodible matrix for controlled release.

The carriers for internal administration can be any carriers commonly used to facilitate the internal administration of compositions such as plasma, sterile saline solution, IV solutions or the like. Carriers for administration through mucus membranes can be any well-known in the art. Carriers for administration oral can be any carrier well-known in the art.

The formulations may be conveniently presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing the active agent into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into the desired formulations.

Formulations suitable for parenteral administration conveniently include a sterile aqueous preparation of the active agent, or dispersions of sterile powders of the active agent, which are preferably isotonic with the blood of the recipient. Isotonic agents that can be included in the liquid preparation include sugars, buffers, and sodium chloride. Solutions of the active agent can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions of the active agent can be prepared in water, ethanol, a polyol (such as glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, glycerol esters, and mixtures thereof. The ultimate dosage form is sterile, fluid, and stable under the conditions of manufacture and storage. The necessary fluidity can be achieved, for example, by using liposomes, by employing the appropriate particle size in the case of dispersions, or by using surfactants. Sterilization of a liquid preparation can be achieved by any convenient method that preserves the bioactivity of the active agent, preferably by filter sterilization. Preferred methods for preparing powders include vacuum drying and freeze drying of the sterile injectible solutions. Subsequent microbial contamination can be prevented using various antimicrobial agents, for example, antibacterial, antiviral and antifungal agents including parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. Absorption of the

active agents over a prolonged period can be achieved by including agents for delaying, for example, aluminum monostearate and gelatin.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as tablets, troches, capsules, lozenges, wafers, or cachets, each containing a predetermined amount of the active agent as a powder or granules, as liposomes containing the active agent, or as a solution or suspension in an aqueous liquor or non-aqueous liquid such as a syrup, an elixir, an emulsion, or a draught. The amount of active agent is such that the dosage level will be effective to produce the desired result in the subject.

Nasal spray formulations include purified aqueous solutions of the active agent with preservative agents and isotonic agents. Such formulations are preferably adjusted to a pH and isotonic state compatible with the nasal mucous membranes. Formulations for rectal or vaginal administration may be presented as a suppository with a suitable carrier such as cocoa butter, or hydrogenated fats or hydrogenated fatty carboxylic acids. Ophthalmic formulations are prepared by a similar method to the nasal spray, except that the pH and isotonic factors are preferably adjusted to match that of the eye. Topical formulations include the active agent dissolved or suspended in one or more media such as mineral oil, DMSO, polyhydroxy alcohols, or other bases used for topical pharmaceutical formulations.

Useful dosages of the active agents can be determined by comparing their *in vitro* activity and the *in vivo* activity in animal models. Methods for extrapolation of effective dosages in mice, and other animals, to humans are known in the art; for example, see U.S. Patent No. 4,938,949.

The tablets, troches, pills, capsules, and the like may also contain one or more of the following: a binder such as gum tragacanth, acacia, corn starch or gelatin; an excipient such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; a sweetening agent such as sucrose, fructose, lactose or aspartame; and a natural or artificial flavoring agent. When the unit dosage form is a capsule, it may further contain a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or

to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac, or sugar and the like. A syrup or elixir may contain one or more of a sweetening agent, a preservative such as methyl- or propylparaben, an agent to retard crystallization of the sugar, an agent to increase the solubility of any other ingredient, such as a polyhydric alcohol, for example glycerol or sorbitol, a dye, and flavoring agent. The material used in preparing any unit dosage form is substantially nontoxic in the amounts employed. The active agent may be incorporated into sustained-release preparations and devices.

EXAMPLES

The invention will be further described by reference to the following detailed examples. The examples are meant to provide illustration and should not be construed as limiting the scope of the present invention.

Examples 1-5: Materials and Methods

Cell cultures: Pheochromocytoma (PC12) cells were provided by Dr. Regino Perez-Polo (University of Texas Medical Branch, Department of Human Biological Chemistry and Genetics) and maintained in EMEM supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 micrograms per milliliter (μg/ml)). Exponentially growing populations of PC12 cells were sub-cultured and used for all experiments.

Western blot analysis: PC12 cells were plated at 7 x 10^6 cells/T75 flask. After exposure to 4HNE, colostrinin or their combination, cells were collected and lysed in 50 millimolar (mM) Tris, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 10% glycerol and protease inhibitor cocktail (supplemented with 1 mM Na_3VO_4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at 14,000g for 10 minutes (min) (4°C) and 40 μg of protein was fractionated on a 10% SDS-polyacrylamide gel and transferred to protein-optimized membranes (Amersham, Inc.). p53 was detected using specific antibody (DO1; Santa Cruz Biotechnology, Inc.) at a dilution of 1:300.

Adducts were detected using an antibody to HNE-protein adducts (Pharmingen, Inc.) at a dilution of 1:500. The anti-phospho-JNK antibody (New England Biolabs, Inc., Beverly, MA) was raised against a synthetic phosphopeptide (SFMMT*PY*VVTRYYR) corresponding to residues 179-193 of JNK. For visualization of primary antibody binding, all blots were incubated with horseradish peroxidase-conjugated secondary antibody (Amersham, Inc.) at a dilution of 1:2000, followed by chemiluminescence detection (Amersham, Inc.) and autoradiography.

Immunocytochemistry: PC12 cells grown on cover-slips were fixed overnight in PBS containing 2% paraformaldehyde at 4°C. Cells were permeabilized by 0.3% Triton X-100, washed in PBS then incubated with primary antibody in PBS containing 0.05% Tween 20 (PBS-T). After washing 3 times in PBS-T, FITC-labeled anti-rabbit IgG (Santa Cruz Biotechnology Inc.) was added. Cells were washed (5 times, for 10 min) with PBS-T and mounted on microscope slides in anti-fade solution (Dako, Inc.). Images of cellular immunofluorescence were acquired using a NIKON Eclipse TE300 scanning microscope.

Measurement of glutathione (GSH): In brief, PC12 cells were mock- or pre-treated with CLN or lactalbumin hydrolysate (LAH), both in 10 μg/ml concentration and then exposed to 4HNE (25 nM). PBS-washed (twice) cells were then extracted with 25% (w/v) metaphosphoric acid solution containing 5 mM EDTA. After ultracentrifugation (105,000g for 30 min), 100 μl of 100 mM phosphate solution (pH 8.0) containing 5 mM EDTA and 10 μl of *o*-phthalaldehyde OPA(OPD; Molecular Probes, Inc.) was added to the supernatant, and the fluorescence intensity at 420 nm determined with excitation set at 350 nm (A.P. Senft et al., *Anal Biochem.*, 280:80-86 (2000)).

Flow cytometry: Relative changes in ROS levels were determined as described previously (I. Boldogh et al., *Psychogeriatr Ann*, 4:57-65 (2001)). Briefly, PC12 cells at 70% confluence were trypsinized and washed with EMEM containing 10% FBS. Cells were re-suspended in EMEM (plus 5 %

FBS) and loaded with 2',7'dichlorodihydro-fluorescein diacetate (H_2DCF-DA; Molecular Probes Inc.) (5 mM final concentration) for 15 min, at 37°C then washed in growth medium. Following centrifugation, the cell pellets were re-suspended in EMEM containing 10 mM HEPES (pH: 7.4). DCF-mediated fluorescence of treated and mock-treated cells was determined by flow cytometry (Becton Dickinson FACS Scan) using 488 nm and 525 nm excitation and emission settings, respectively. Each data point represents the mean fluorescence for 12,000 cells.

Reagents: Colostrinin (CLN) was purified from ovine colostrum, collected during the first milking (6-12 hours (hr) after lambing), according to the method developed by Janusz et al. (M. Janusz et al., *FEBS Lett.*, 49:276-279 (1974)). A high content of proline (>23 %) and lack of detectable alanine, arginine, histidine, tryptophan, methionine, and cysteine were confirmed by amino acid analysis of CLN. A peptide control was prepared by trypsin (Sigma-Aldrich) digestion of purified lactalbumin from bovine milk (Sigma). The trypsin was then inhibited by treatment with inhibitor (Invitrogen). SDS-PAGE confirmed digestion of lactalbumin into peptides, and the hydrolysate was referred to as LAH.

Statistical analysis: The experiments were repeated at least three times and statistically analyzed for significant differences using ANOVA procedures and Student's t-tests. Data are expressed as means ±S.E.

Examples 1-5: Results

Example 1: Colostrinin reduces 4HNE-protein adduct formation in PC 12 cells.

Fluorescent microscopy and Western blot analysis was undertaken to investigate the extent of 4HNE protein-adduct formation in cultures of PC12 cells in the presence of CLN. Cells were pretreated with CLN or LAH in the presence or absence of 4HNE and then analyzed for the formation of 4HNE-protein adducts. The results in Fig. 1A and 1B show that addition of 4HNE

(25 nanomolar (nM)) or H_2O_2 (100 micromolar (μM)) resulted in a bright fluorescence, localized to the cytoplasmic region of PC12 cells due to binding of antibody to 4HNE-protein adducts. When cells were pre-treated with CLN (10 microgram per milliliter (μg/ml)) for 15 minutes (min) and exposed to 4HNE (25 nM) for 15 min (concentrations of CLN and time required for effect were determined in preliminary studies), the results indicated that CLN reduced fluorescence intensity (Fig. 1C) to background level (data not shown). In the controls, pre-treatment of cells with an N-acetyl-L-cysteine (10 mM) and trolox (1 mM; a water-soluble -tocopherol) combination significantly reduced 4HNE-mediated intracellular fluorescence.

To determine whether the inhibitory effect of CLN was specific, CLN was substituted with digested lactalbumin hydrolysate (LAH, Materials and Methods), which contains a variety of peptides as does CLN. Results in Fig. 1D show bright fluorescence in cells treated with LAH (10 μg/ml) plus 4HNE (25 nM), which is similar to that seen with 4HNE alone (Fig.1A). These data indicate that CLN inhibits adduct formation, and the effect is specific and could be the result of a not yet-determined interaction between its constituent peptides and cellular component(s).

To confirm the results generated by immunochemistry, Western blot analysis was used to investigate changes in 4HNE-protein adduct levels in cells treated with 4HNE alone or CLN plus 4HNE. Figure 1E (lanes 1, 2, and 3) shows that 4HNE alone induced a significant increase in levels of 4HNE-protein adducts, with molecular weights ranging from 200 kD to 15 kD. CLN (10 μg/ml) abolished adduct formation, as shown in Fig. 1E lanes 4 to 6. Overall these data indicate that CLN can block the formation of 4HNE-adducts. From these results, it is believed that the inhibition of 4HNE-protein adduct formation by CLN is multi-factorial and may involve mechanisms such as direct scavenging (binding) of 4HNE via cysteine, lysine, or histidine residues in CLN, or by inhibition of 4HNE's entry onto cells.

To determine whether CLN can protect mitochondria and abolish the oxidative stress induced by 4HNE, PC12 cells were treated with CLN (with LAH as control) and/or 4HNE and the changes in ROS levels were monitored by the redox-sensitive 2',7'-dichlorofluorescein diacetate (H_2DCF-DA) probe

(I. Boldogh et al., *Psychogeriatr Ann.*, 4:57-65(2001); LeBel, *Chem. Res. Toxicol.*, 5:227-231 (1992)). Mock- as well as CLN (or LAH) pre-treated cells were loaded with H_2DCF-DA then exposed to 4HNE for 15 min. Changes in fluorescence intensities mediated by the oxidized probe, DCF, were determined by flow cytometry. Prior to data collection, propidium iodide was added to the samples for sorting out nonviable cells.

Example 2: Colostrinin affects the oxidative metabolism in PC12 cells.

A representative histogram showing the effects of the treatments on ROS levels is shown in Fig. 2B. As summarized in Fig. 2A, 4HNE (25 nM) induced a 4- to 5-fold increase in DCF-mediated fluorescence, while CLN alone or LAH showed no significant effect. Remarkably, CLN abolished (while LAH had no significant effect on) H_2DCF oxidation in 4HNE-treated PC 12 cells. Because constituent peptides in LAH did not alter 4HNE-induced H_2DCF oxidation, it can be concluded that the effect of CLN is specific, and may protect cells from ROS damage via its quantitatively unique and specific peptide composition.

Example 3: Effect of CLN on 4HNE –induced loss of intracellular GSH levels.

To investigate whether the anti-oxidant effect of CLN was due to protection of intracellular GSH levels, PC12 cells were pre-treated (with CLN or LAH) and exposed to 4HNE, as described above, and changes in GSH levels were determined fluorimetrically. The results summarized in Fig. 3 show that treatment with 4HNE alone for 30 min (time determined in preliminary studies) resulted in a significant reduction of intracellular GSH levels as shown by a change in OPA-GSH's fluorescence. OPA (o-phthalaldehyde or phthalic dicarboxaldehyde) is highly fluorescent when it is conjugated to GSH (A.P. Senft et al., *Anal Biochem.*, 280:80-86 (2000)). Pre-treatment of cells with CLN (10 μg/ml), however, significantly inhibited this change in OPA fluorescence (loss of GSH) while LAH had an insignificant effect.

To determine whether the loss of intracellular GSH was due to it's extrusion from the cells or oxidation, the level of reduced GSH in the extra-cellular fluid was evaluated. Relative to CLN- or mock-treated cells, 4HNE

caused a significant increase in OPA-GSH-mediated fluorescence when it was added to extracellular fluid (data not shown). LAH alone or LAH in 4HNE-exposed cells did not affect GSH extrusion (OPA-GSH fluorescence). OPA did not show fluorescence when it was mixed with CLN, LAH or 4HNE alone. These results indicate that CLN mediates its effect on GSH metabolism at the cell membrane level.

Example 4: 4HNE-induced activation of JNK is suppressed by CLN.

The effect of CLN on 4HNE-induced activation of JNK in PC12 cells was investigated. The activation of JNK was monitored by Western blot analysis using a highly specific anti-phospho- (Thr-183/Tyr-185) JNK antibody (Materials and Methods).

The data summarized in Fig. 4 show that 4HNE alone is a potent inducer of JNK phosphorylation (Fig.4, lanes 4 and 5). Pretreatment of PC12 cells with CLN (1 and 10 μg/ml) or with an inhibitor of JNK activation [8-(4-chlorophenylthio)-cAMP] prevented 4HNE-induced JNK phosphorylation; 4HNE-mediated phosphorylation was reduced by 10 and 1.0 μg/ml CLN (Fig. 4, lanes 7 and 8) to control levels (Fig.4 lanes 1 and 2). CLN at 0.1 μg/ml concentration did not significantly effect 4HNE-mediated JNK phosphorylation (lane 9). The maximum level of phosphorylation of JNK in 4HNE-treated cells occurred between 15 and 30 min post-treatment as determined in preliminary studies (data not shown). These data indicate that CLN may modulate oxidative metabolism (GSH levels, 4HNE-protein adduct formation) of cells potentially through JNK, a kinase that is central to the cellular stress responses.

Example 5: Colostrinin inhibits 4HNE-induced activation of p53.

Whether CLN could modulate p53 levels after 4HNE exposure was also investigated. In Figure 5, Western blot analysis shows that CLN reduces activation of p53 induced by 4HNE when compared to cells treated with 4HNE alone. On the other hand, pre-treatment with the same concentration (10 μg/ml) of LAH had no affect on 4HNE-mediated p53 induction. These data suggest that CLN, via its antioxidant activity, can effect activation of p53,

a key regulator of cell proliferation, differentiation and apoptosis (G. Evan et al., *Science*, 281:1317-1322 (1998)) and may explain multiple biological effects (antioxidant, differentiation) of CLN.

Examples 1-5: Discussion

It has been shown that CLN, a milk-derived peptide complex can modulate both cytokine production and cellular redox status. To study CLN's antioxidant effects, 4HNE was used for treatment of PC12 cells. 4HNE is a 3-unsaturated aldehyde generated endogenously during lipid peroxidation, specifically from the oxidative degradation of arachidonic and linoleic acids (H. Esterbauer et al., *Free Radic. Biol. Med.*, 11:81-128 (1991)). Further, 4HNE is involved in both normal and pathophysiological events in cells and tissues that result in various chronic diseases. While micromolar concentrations of 4HNE is cytotoxic, at the nanomolar level it can be involved in activation of the signal transduction pathways. For example, it has been shown that depending on concentration, 4HNE can affect proliferation and induce differentiation or apoptosis in cells.

In the current studies, a concentration of 25 nM 4HNE was used. This did not show toxic effects or induce apoptosis but considerably increased the levels of 4HNE-protein adducts (Fig. 1A) in PC12 cells. Remarkably, it was found that CLN abolished 4HNE-protein adduct formation, while LAH (as control) at the same concentration had no effect indicating CLN's specificity. Due to the presence of a highly electrophilic carbon, 4HNE is a potent alkylating agent able to react with histidine, lysine, serine, cystein, and tyrosine side chains in proteins, and thus modify their functions. Although it was hypothesized that peptides of CLN, via its component amino acid residues, were reacting with 4HNE and chemically reducing its concentration, no direct interaction between CLN's peptide(s) and 4HNE were observed.

4HNE is known to modulate the activities of ATPases, phospholipase C, adenylate cyclase, GTP-binding proteins, and protein kinase C. Furthermore, 4HNE can react with the nucleophilic sites in DNA, mitochondrial proteins and a variety of other nucleophiles, including GSH, resulting in cellular stress responses and oxidative stress. In the present

studies, it was demonstrated that that CLN was able to prevent a decrease in 4HNE-induced GSH levels. It is proposed that 4HNE-induced reduction in GSH levels may be due to glutathione-S-transferase (GST)-mediated conjugation of 4-HNE to GSH, or that GSH may be utilized in detoxification reactions of ROS.

Taking into consideration that only 25 nM of 4HNE was used, while intracellular concentrations of GSH are in the 0.5 to 10 mM range, reduction of GSH levels by GST or utilization by glutathione peroxidases may not explain the more than 50% loss of GSH. Therefore, the GSH levels in the extracellular fluid was evaluated. The large reduction in GSH levels (Fig. 3) may be due to extrusion of GSH from cells after 4HNE exposure. Indeed, it has been discovered that an increase in GSH in the extracellular milieu is in response to 4HNE treatment. Most remarkably, CLN was able to prevent this effect of 4HNE.

Although some ROS production in 4HNE-treated cells has been shown to be due to mitochondrial damage, it is believed that the 3- to 4-fold increase in ROS levels were due to GSH extrusion, which resulted in a perturbance of cellular anti-oxidant defenses. Most importantly, CLN, but not LAH, inhibited oxidation of H_2DCF strongly suggesting that CLN is involved in the activation of cellular antioxidant defenses or possesses effective anti-oxidant activity via regulating cellular GSH levels.

4HNE exposures have been reported to be linked with c-Jun NH2-terminal kinases activation and c-Jun phosphorylation. Three groups of mitogen-activated protein (MAP) kinases have been identified in mammals: the extracellular signal-regulated kinase, the p38 MAP kinase, and JNKs (also referred to SAPKs). JNKs are activated by a wide variety of stimuli, including ROS, DNA-damaging agents and inhibitors of protein synthesis, and heat or osmotic shock. These stimuli appear to operate through small G proteins of the Ras and epidermal growth factor (EGF) family receptors and sequential activation of various protein kinases. Targets of the JNK signal transduction pathway include the transcription factors ATF2 and c-Jun. c-Jun binds to the N-terminal region of ATF2 and c-Jun and phosphorylates two sites within the activation domain. These factors are members of the basic leucine zipper

group that binds as homo- and heterodimeric complexes to AP-l and AP-l-like sites in the promoters of many genes and result in increased transcriptional activity.

The present studies show that treatment of PC12 cells with 4HNE causes JNK activation within 15 to 30 min. However, in CLN pre-treated cells JNK activation was not only delayed or reduced, it was abolished. CLN was also as potent as 8-(4-chlorophenylthio)-cAMP a specific inhibitor of JNK activation. AP-l phosphorylation, which is a later event in the JNK signaling pathway is presently under investigation. These findings are consistent with the idea that CLN has the ability to protect cells from oxidative stress and other consequences of 4HNE exposure, including JNK activation, and its down-stream consequences.

The Western blot analysis shown in Fig. 5, clearly demonstrated that p53 is normally present in a latent form and that 4HNE induced its activation. It has been shown that p53 lies at the center of a network of complex redox interactions. In this network, p53 can control the timely production of ROS, but this activity is itself under the control of changes in cellular redox status. Thus, p53 activation in 4HNE-treated PC12 cells can occur in multiple ways: it may be due to 4HNE-induced DNA damage, ROS, and/or activation of cell cycle regulatory kinases. Regardless of the mechanism of p53 activation, CLN showed a potent inhibitory effect.

CLN induced differentiation in SH-SY5Y cells in a dose dependant manner (Table 1A). The ability of CLN to induce differentiation in these cells is shown in Figure 6A, the control compared to Figure 6C, a culture treated with 0.1 μg/ml CLN (see figure legends).

It has been shown that colostrinin can inhibit the toxicity of Beta-amyloid in neural derived SH-SY5Y cells. Essentially complete inhibition of the toxicity occurred at the 0.01 μg/ml level (Table 1B). Figures 6D and 6E shows the protective effect of 3.0 and 0.1 μg/ml of CLN on B-amyloid induced toxicity as shown in Figure 6B. Since this toxicity is the result of the apoptotic activity of Beta-amyloid (β-amyloid), the data indicate that colostrinin is a potent inhibitor of apoptosis in neural-derived cells. This potent activity indicated that even lower concentrations of colostrinin would

have to be tested to determine the potency and anti-apoptotic dose response effect of colostrinin in this system. However, a dose dependant development of differentiation did occur in the presence of Beta-amyloid in the colostrinin treated cells (Figures 6D and 6E).

The results indicate that not only did colostrinin inhibit the toxicity of Beta-amyloid, but it also was able to induce differentiation of the SH-SY5Y in a dose dependant manner in Beta-amyloid treated cells. This finding indicates that the development of differentiation in Beta-amyloid treated cells could be used as a biological assay for colostrinin and one of its important functions.

To determine whether this was reproducible and to determine the potency of CLN to inhibit retinoic acid toxicity, two concentration of retinoic acid were used to treat cells on day one of the experiment, 20 μM and 40 μM. *Control wells were mock-treated for the duration of the experiment. Retinoic Acid was left on the plate for the duration of the experiment or until washed off. Colostrinin was added to the plate at the indicated doses. When added on Day 1, it was present during the entire experiment. When added on Day 5, it was incubated on the plate for 30 minutes at 37°C and then removed. The wells were washed twice with PBS before adding β-Amyloid. Cultures were then observed under the microscope and graded.

The data reported herein shows the ability of CLN to block the activity of cells treated with 20 μM retinoic acid (the toxicity developed too rapidly in cells treated with 40 μM). Table 1C indicates that 1.0 μg/ml of CLN almost completely blocked the cytotoxicity of retinoic acid. The retinoic acid induced differentiation in these cells by day two, but the cells started showing signs of toxicity by day 6. CLN, 1.0 μg/ml, added on day one or for 30 minutes on day five of the experiment completely blocked the toxicity, and similar to the finding with Beta-amyloid, also induced the cells to proliferate. Figures 6F and 6G further document the finding. Figure 6F shows the toxicity induced by retinoic acid compared to Figure 6G, which clearly shows differentiated cells (see legends).

Colostrinin inhibited the eventual development of cytotoxicity by Retinoic Acid when added at the same time or 5 days later. Colostrinin added at Day 1 also inhibited the development of toxicity by β-Amyloid added on Day 5 and was dose dependent (data not shown).

The ability of CLN to inhibit apoptotic effects of two substances, Beta-amyloid and retinoic acid, indicates it may have potential biological use in many areas where apoptosis plays a role, e.g., virus infections, chronic diseases and attempts to get stem cells to grow and differentiate, among many others.

Table 1. Differentiating, Anti-Apoptotic and Protective Activity of Colostrinin Against β-Amyloid and Retinoic Acid in Neuronal Derived SH-SY5Y Cells

TREATMENT	DOSE COLOSTRININ (MG/ML)	TOXICITY	DIFFERENTI-ATION
A. Differentiating Activity			
	3.0 *(Day 5)*[a]	– *(Day 8)*[b]	++++ *(Day 8)*[b]
	1.0 *(Day 5)*[a]	– *(Day 8)*[b]	++++ *(Day 8)*[b]
	0.1 *(Day 5)*[a]	– *(Day 8)*[b]	+++ *(Day 8)*[b]
	0.01 *(Day 5)*[a]	+/– *(Day 8)*[b]	++ *(Day 8)*[b]
B. Anti-apoptotic Activity			
β-Amyloid 10μg/ml *(Day 5)*[a]		+++ *(Day 8)*[b]	– *(Day 8)*[b]
β-Amyloid 10μg/ml *(Day 5)*[a]	3.0 *(Day 5)*[a]	– *(Day 8)*[b]	+++ *(Day 8)*[b]
"	1.0 *(Day 5)*[a]	+/- *(Day 8)*[b]	+++ *(Day 8)*[b]
"	0.1 *(Day 5)*[a]	+/- *(Day 8)*[b]	++ *(Day 8)*[b]
"	0.01 *(Day 5)*[a]	+ *(Day 8)*[b]	+ *(Day 8)*[b]
C. Protection Against Retinoic Acid Activity			
Retinoic Acid 20μM *(Day 1)*[a]		– *(Day 2)*[b]	++++ *(Day 2)*[b]
Retinoic Acid 20μM *(Day*		+++ *(Day 8)*[b]	+ *(Day 8)*[b]

1)[a]			
Retinoic Acid 20μM *(Day 1)*[a]	1.0 *(Day 5)*[a]	– *(Day 8)*[b]	++++ *(Day 8)*[b]
"	1.0 *(Day 1)*[a]	+ *(Day 8)*[b]	++++ *(Day 8)*[b]
D. Control			
Mock Treated *(Day 1)*[a]		- *(Day 5 & 8)*[b]	– *(Day 5 & 8)*[b]

++++ Approximately 100% of the cells

+ Approximately 25% of the cells

[a] Day of treatment

[b] Day cells observed

Example 7: Inhibition of 4HNE-induced apoptosis by CLN.

Apoptosis is a specific mode of cell death recognized by a characteristic pattern of morphological, biochemical, and molecular changes. Currently the hallmark of apoptosis *in vitro* is DNA fragmentation and changes in plasma membrane reorganization that allows for the surface expression of phosphatidyl-D-serine and result in increased membrane permeability. The protection of cells against 4HNE by CLN using increased permeability and cell membrane expression of phosphatidyl-D-serine was investigated.

Cells were simultaneously labeled with fluorochrome-conjugated annexin V-PE (detecting PS asymmetry in the plasma membrane, an early marker of apoptosis), 7-aminoactinomycin D (7-AAD) (detecting increased membrane permeability associated with both apoptosis and necrosis). A dual laser flow cytometer (either a Becton-Dickinson FACScan) was used for the simultaneous detection of the PE-conjugated annexin V (which is excited at 632 nm and emits at 660 nm), 7-AAD (excited at 488 nm and emitting at 670 nm). Fluorochrome compatibility was excellent, although careful intralaser compensation is required for simultaneous use of PE and 7-AAD.

The analysis of apoptosis by flow cytometry is shown in Figure 7. The results of the inhibition of 4HNE-induced apoptosis by CLN are shown in Figure 8.

Example 8: Inhibition of UV-irradiation-induced apoptosis by CLN.

Chronic repeated UV exposures are the primary cause of benign and malignant skin tumors, including malignant melanoma. In experimental animal models, among types of solar radiation, ultraviolet B (290-320 nm) radiation is highly mutagenic and carcinogenic compare to ultraviolet A (320-400 nm) radiation. Based on current understanding of DNA damage caused by direct UV radiation and by indirect stress via reactive oxygen species and DNA repair mechanisms are responsible for UV irradiation-induced skin tumor development in human cells.

UVB exposure leads to a time-dependent increase in the production of intracellular peroxide and superoxide anions and may induce carcinogenic mutations and apoptosis. Besides being a major cause of oxidative stress in the cells, UVB-irradiation induces apoptosis by a large number of unrelated pathways such as enhanced Fas transcription and/or mRNA stability, induction of transcriptional factors viz c-fos, c-jun, SAP-1 and nuclear factor kB gene expression. A possible prevention of UV-induced skin cancer by feeding or topical use of antioxidants, such as polyphenols, and vitamins are observed.

The effect of CLN on ultraviolet B (UVB)-induced apoptosis and DNA damage in cultured PC12 cells has been determined. The apoptosis was determined by flow cytometry. The comet assay was employed to detect DNA damage in individual cell. The results shown in Figure 9 indicate an inhibitory effect of CLN on UVB-induced apoptosis. CLN-treated cells also showed a significantly reduced DNA damage. Semi-confluent cells with >98% viability (tested with trypan blue dye exclusion) were used in all experiments. PC12 cells were exposed to UV-B irradiation. Lethal dose 50 (LD50) was determined in preliminary studies. Cells were irradiated by a dose result in 50% cell death.

CONCLUSION

Taken together, these results show that CLN can be involved in regulation of the cellular redox status, GSH metabolism, and modulation of ROS-induced signaling-mediated down-stream events (e.g., JNK, p53). These

results further suggest a potential mechanism(s) by which CLN could modulate a network, resulting in cytokine, chemokine production, cell differentiation and may explain it's beneficial effects on pathogenic processes involved in AD and other chronic neuro-degenerative diseases.

Although the invention has been disclosed with reference to its preferred embodiments, from reading this description those of skill in the art may appreciate changes and modification that may be made which do not depart from the scope and spirit of the invention as described above and claimed hereafter. All references, patents, and patent applications cited herein are incorporated herein by reference in their entirety as if individually incorporated.

Sequence Listing Free Text

The following are all synthetic peptide sequences.

SEQ ID NO:1	MQPPPLP
SEQ ID NO:2	LQTPQPLLQVMMEPQGD
SEQ ID NO:3	DQPPDVEKPDLQPFQVQS
SEQ ID NO:4	LFFFLPVVNVLP
SEQ ID NO:5	DLEMPVLPVEPFPFV
SEQ ID NO:6	MPQNFYKLPQM
SEQ ID NO:7	VLEMKFPPPPQETVT
SEQ ID NO:8	LKPFPKLKVEVFPFP
SEQ ID NO:9	VVMEV
SEQ ID NO:10	SEQP
SEQ ID NO:11	DKE
SEQ ID NO:12	FPPPK
SEQ ID NO:13	DSQPPV
SEQ ID NO:14	DPPPPQS
SEQ ID NO:15	SEEMP
SEQ ID NO:16	KYKLQPE
SEQ ID NO:17	VLPPNVG
SEQ ID NO:18	VYPFTGPIPN

	SEQ ID NO:19	SLPQNILPL
	SEQ ID NO:20	TQTPVVVPPF
	SEQ ID NO:21	LQPEIMGVPKVKETMVPK
	SEQ ID NO:22	HKEMPFPKYPVEPFTESQ
5	SEQ ID NO:23	SLTLTDVEKLHLPLPLVQ
	SEQ ID NO:24	SWMHQPP
	SEQ ID NO:25	QPLPPTVMFP
	SEQ ID NO:26	PQSVLS
	SEQ ID NO:27	LSQPKVLPVPQKAVPQRDMPIQ
10	SEQ ID NO:28	AFLLYQE
	SEQ ID NO:29	RGPFPILV
	SEQ ID NO:30	ATFNRYQDDHGEEILKSL
	SEQ ID NO:31	VESYVPLFP
	SEQ ID NO:32	FLLYQEPVLGPVR
15	SEQ ID NO:33	LNF
	SEQ ID NO:34	MHQPPQPLPPTVMFP